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Introduction

The overall goal of the project is to explore the role of protein elongation factor eEF1A2 in breast tumour development and to determine whether eEF1A2 is a useful breast cancer prognostic factor. Elongation factor eEF1A2 is one of two members of the eEF1A family of proteins (eEF1A1 and eEF1A2) that bind amino-acylated tRNA and facilitate their recruitment to the ribosome during protein translation elongation[1]. eEF1A proteins have other functions and can also induce actin [2] and tubulin [3] cytoskeleton rearrangements. Inactivation of the mouse eEF1A2 homolog, *Eef1a2*, leads to immunodeficiency and neural/muscular defects and death by 30 days of age [4, 5]. We had previously identified eEF1A2 as an ovarian cancer oncogene that could transform human and mouse cells[6], but its role in breast cancer was unknown. We also proposed to test the idea that eEF1A2 could modulate sensitivity to cisplatin and taxol and whether eEF1A2-inactivation could be used as a treatment for breast cancer. In addition, we hoped to understand the mechanism by which eEF1A2 regulates cell and oncogenesis.

We have made progress in the following areas:

1. The prognostic significance of eEF1A2 in breast cancer.
2. The ability of eEF1A2 to enhance the growth properties of malignant breast cells.
3. Generation of eEF1A2 transgenic mice.
4. The role of eEF1A2 in regulating the cytotoxicity of anti-cancer agents.
5. eEF1A2 inactivation as an anti-cancer treatment.
6. Modulation of cell adhesion and migration by eEF1A2.

1. The prognostic significance of eEF1A2 in breast cancer.

Progress. We have made progress in two overall areas: a) Correlation of eEF1A2 expression and gene amplification with clinical and histological parameters; b) Creation of an eEF1A2 antibody to correlate eEF1A2 protein expression with clinical and histological parameters. For the first aim, we analyzed 69 primary breast tumours with recurrence annotation for eEF1A2 expression and divided the samples into eEF1A2 positive and negative groups (Fig 1a). eEF1A2 mRNA expression does not correlate with tumour size (Fig 1b), but women whose tumour was eEF1A2 positive had an increased probability of 48-month breast cancer tumour recurrence ($p<0.01$) relative to the eEF1A2 negative group (Fig 1c). In addition, Fig. 1d shows that increased eEF1A2 gene (*EEF1A2*) copy number correlates with significantly ($p<0.05$) decreased 10-year survival (n=142). Taken together, this suggests that eEF1A2 is a useful breast cancer5 prognostic marker. A paper containing this and other data has been submitted (Jeganathan *et al*, see Appendix).

To complement the eEF1A2 gene and mRNA studies, we have derived a rabbit polyclonal antibody that is reactive against eEF1A2. This antibody recognizes eEF1A2 in Western blots of whole cell lysates from breast cells infected with an eEF1A2 adenovirus (Fig 2a) and whole cell lysates of MCF7, a breast line that expresses abundant endogenous eEF1A2 mRNA (Fig 2b). The antiserum recognizes a GST-purified eEF1A2 and eEF1A2 siRNA reduce the abundance of the recognized protein (not shown). This antibody can recognize eEF1A2 in paraffin-embedded eEF1A2-expressing cells. eEF1A2 protein expression is now being tested in a large breast cancer tumour micro-array for correlation with histological and survival parameters in breast cancer.

2. eEF1A2 enhances growth properties of human malignant breast cell lines.

Progress. We have analyzed eEF1A2 in a panel of breast cancer cell lines and identified lines that highly express eEF1A2 and those that do not (Fig. 3a). We have made variants of MCF10AT and BT549 cell lines that highly express eEF1A2 (Fig 3b). eEF1A2 expression increases the *in vitro* growth rates of both cell lines (Fig 3c). In addition, eEF1A2 expression increases the resistance of BT549 cell lines to anoikis, apoptosis induced by detachment from a solid growth substrate (Fig 4a). Importantly, we have found that eEF1A2 is an activator of the Akt kinase as measured by an eEF1A2-dependent increase in phosphorylation on Akt residues 308 and 473 (Fig 4b). Akt is a well-characterized regulator of cell growth and tumorigenesis. The involvement of eEF1A2 in increasing Akt phosphorylation suggests a plausible mechanism by which eEF1A2 could enhance oncogenesis. A paper containing our observation that eEF1A2 is a novel Akt activator and anoikis inhibitor has been submitted (Jeganathan *et al*, see Appendix).

3. eEF1A2 transgenic mice.

Progress. We have derived three independent lines of mice that express eEF1A2 under the control of the MMTV promoter. Expression of transgenic eEF1A2 in the two highest expressing lines are shown in figure 5a. Line 1 has expression in breast only, while line 2 has expression in breast and liver. 6-8 month old virgin mice from these two lines show some evidence of nodal hyperplasia and increased ductal branching in their duct network (Fig 5b). The oldest of these mice is 8 months of age, but no spontaneous tumours have been observed in virgin mice. We are currently determining whether multiparous females have an increased development of spontaneous breast tumours.

4. Role of eEF1A2 in modulating sensitivity to taxol and cisplatin.

Progress. Using BT549 and MCF10AT lines expressing eEF1A2, we have found no difference in sensitivity to cisplatin and taxol between eEF1A2 expressing cells and parental and vector controls (fig 6). Moreover, si-RNA mediated inactivation of eEF1A2 in MCF7, a breast tumour line that has high endogenous eEF1A2 levels, has no effect on sensitivity to either agent (not shown).

5. Identify eEF1A2-inactivating agents that inhibit breast tumour growth.

Progress. We have derived two eEF1A2 siRNA that reduce eEF1A2 protein in BT549 cells (expressing exogenous eEF1A2) and eEF1A2 mRNA in MCF7 cells (expressing endogenous eEF1A2) (Fig. 7a). Transient transfection of these siRNA into MCF7 reduce *in vitro* growth rate (Fig 7b) and sensitize them to anoikis (Fig 7c). This suggests that eEF1A2 could be a suitable target for anti-cancer therapy. We are currently deriving MCF7 cells that stably express eEF1A2 siRNA (pSilencer) to determine the effect that eEF1A2 inactivation has on *in vivo* tumorigenicity.

6. eEF1A2 regulates cellular adhesion and migration.

Progress. We have found that eEF1A2 regulates actin cytoskeleton rearrangement and *in vitro* migration. As shown in Fig 8a, BT549 cells that stable express eEF1A2 have

more filopodia-like structures than control cell lines, indicating that eEF1A2 is activating cytoskeletal rearrangement. This alteration in actin structure correlates with an increased ability of eEF1A2 to adhere to solid growth substrates, as measured by an increase in the time necessary to centrifugally remove cells from their growth substrate (fig 8b). Moreover, eEF1A2-expressing cells have reduced migration in wound healing assays relative to controls cells (Fig 8c). We are currently preparing a manuscript detailing these findings.

Key Research Accomplishments

- Determined that eEF1A2 expression is increased in approximately 30% of human breast tumours.
- Identified high eEF1A2 mRNA expression as a marker for breast tumour recurrence.
- Generated a rabbit polyclonal antibody that recognizes eEF1A2 protein.
- Determined that eEF1A2 can enhance the growth rate of malignant breast cells.
- Determined that eEF1A2 is an inhibitor of anoikis.
- Determined that eEF1A2 is a novel activator of the Akt/PKB serine/threonine kinase.
- Generated three independent lines of transgenic mice that expresses eEF1A2 in their mammary tissue.
- Generated siRNA that inactivate eEF1A2 and inhibit the in vitro growth of breast cell lines.
- Determined that eEF1A2 regulates the actin cytoskeleton.

Reportable Outcomes

- eEF1A2 expression is increased in approximately 30% of human breast tumours.
- eEF1A2 mRNA expression is a marker for breast tumour recurrence.
- An eEF1A2 rabbit polyclonal antibody has been derived
- eEF1A2 enhances the growth rate of malignant breast cells.
- eEF1A2 inhibits anoikis.
- eEF1A1 activates the Akt/PKB serine/threonine kinase.
- Three independent lines of eEF1A2 transgenic mice have been derived
- siRNA that inactivate eEF1A2 and inhibit the in vitro growth of breast cell lines have been created.
- eEF1A2 regulates the actin cytoskeleton.

Conclusions

EEF1A2 has been identified as a novel breast cancer oncogene. Supporting this idea are our observations that:

- eEF1A2 expression is increased in approximately 30% of human breast tumours.
- eEF1A2 mRNA expression is a marker for breast tumour recurrence.
- An eEF1A2 rabbit polyclonal antibody has been derived
- eEF1A2 enhances the growth rate of malignant breast cells.
- eEF1A2 inhibits anoikis.
- eEF1A1 activates the Akt/PKB serine/threonine kinase.
- Three independent lines of eEF1A2 transgenic mice have been derived
- siRNA that inactivate eEF1A2 and inhibit the in vitro growth of breast cell lines have been created.
- eEF1A2 regulates the actin cytoskeleton.

Taken together, our observations indicate that eEF1A2 is likely to play a causal role in the development of breast cancer and that it is a likely target for breast cancer therapy.

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Appendix A. Figures.

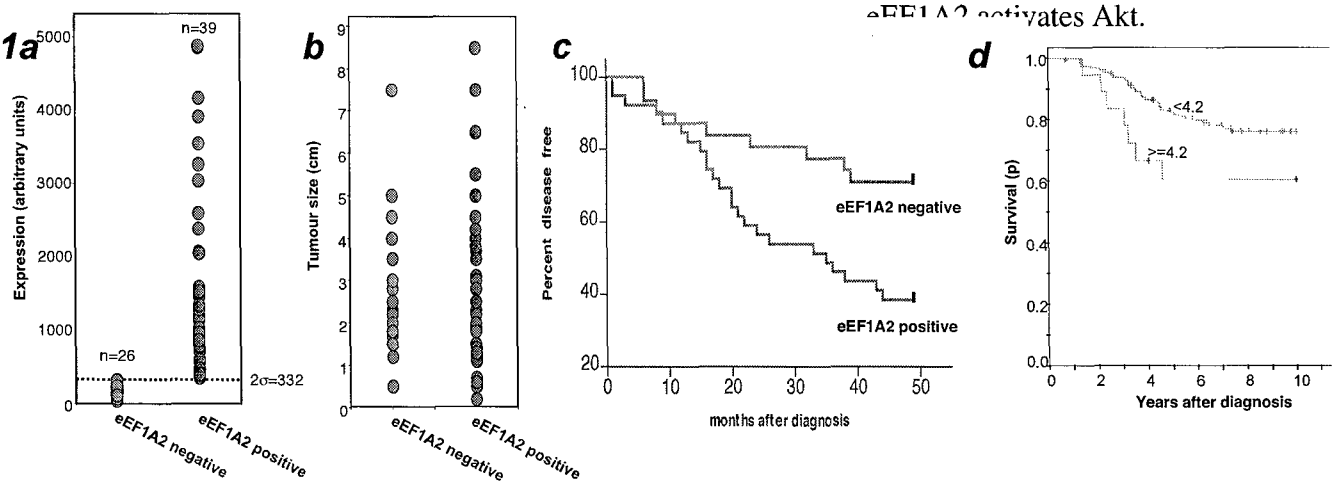


Figure 1. eEF1A2 is a marker for breast tumour recurrence. (*a*) Value of eEF1A2 expression in eEF1A2 positive and negative breast tumours. Th 2σ value above the negative expression is drawn and none of the tumours in the positive population is less than this value. (*b*) Tumour size in the eEF1A2 positive and negative populations. (*c*) The percent disease free faction in the eEF1A2 positive and negative populations was plotted as function of time. Th positive population has a significantly ($p<0.01$) increased probability of recurrence relative to the negative population. (*d*) The percent surviving fraction o women with breast cancer was plotted as a function of time between women whose tumours had more than 4.2 or less than 4.2 *EEF1A2* gene copies. Th women with more *EEF1A2* copies did significantly worse than those with less.

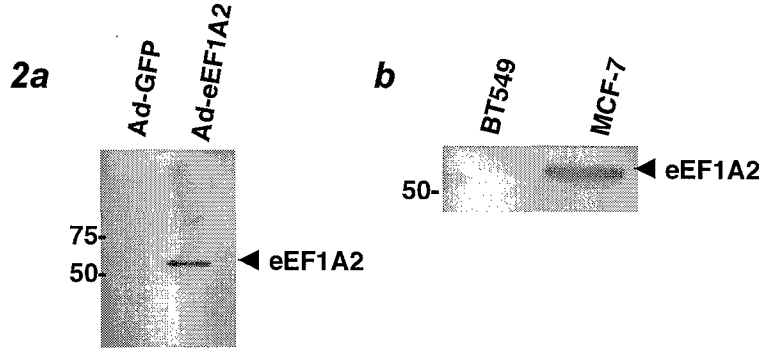


Figure 2. An eEF1A2-specific antibody. (*a*) an eEF1A2-negative cell line (BT549)was infected with an adenovirus for GFP or eEF1A2. 48 hours after infection, whole cell lysates were blotted with an eEF1A2 rabbit polyclonal antiserum. The visualized band is the correct molecular weight for eEF1A2 (~54 kDa). (*b*) Whole cell lysates from an eEF1A2 mRNA negative or positive breast tumour cell line, BT549 and MCF7 respectively, were blotted with an eEF1A2 rabbit polyclonal antiserum.

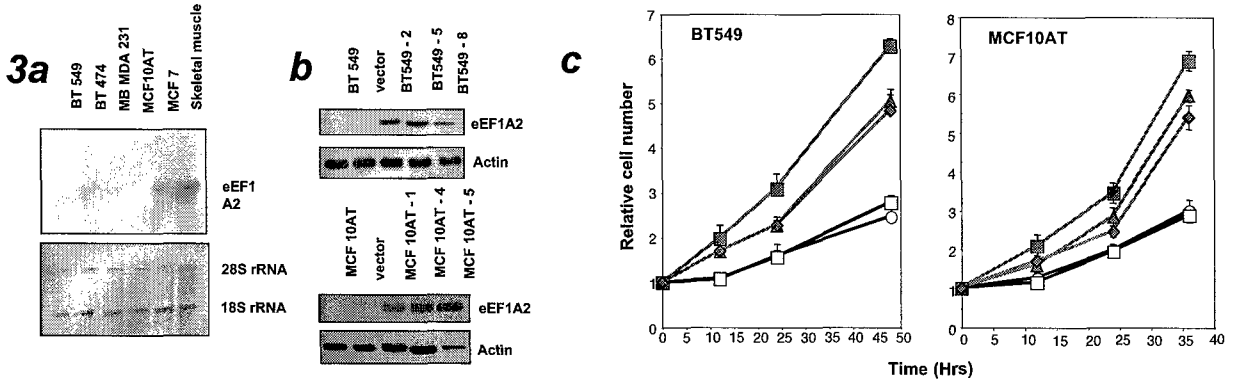


Figure 3. eEF1A2 enhances cell proliferation. (*a*) eEF1A2 mRNA expression, measured by Northern blot in breast cancer cell lines. rRNA staining of the membrane serves as a loading control (*b*) Protein expression of eEF1A2 in independent clones of BT549 and MCF10AT cells, measured by Western blotting. Actin expression serves as a loading control (*c*) Ectopic expression of eEF1A2 leads to increased growth rates in BT 549 and MCF 10AT cell lines. eEF1A2-expressing clones are shown in red symbols, BT-549-2 (▲), BT549-5 (■), BT549-8 (◆), and controls in open symbols, parental (O) and vector alone (□). Points are the mean and standard deviation of triplicate measurements

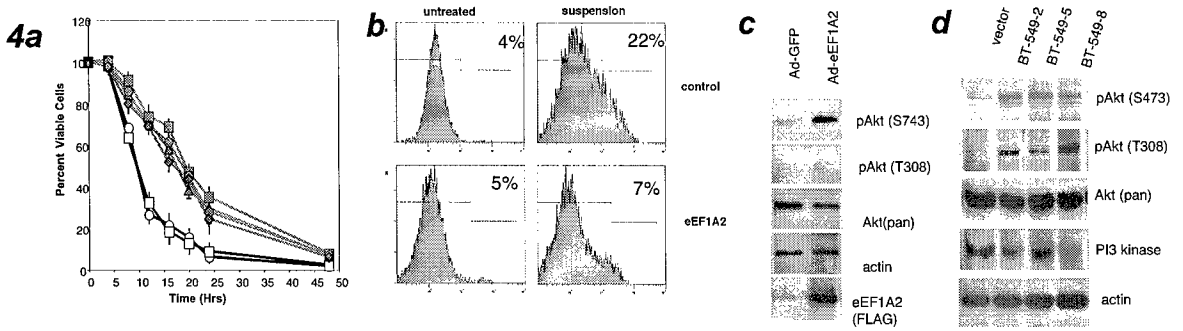
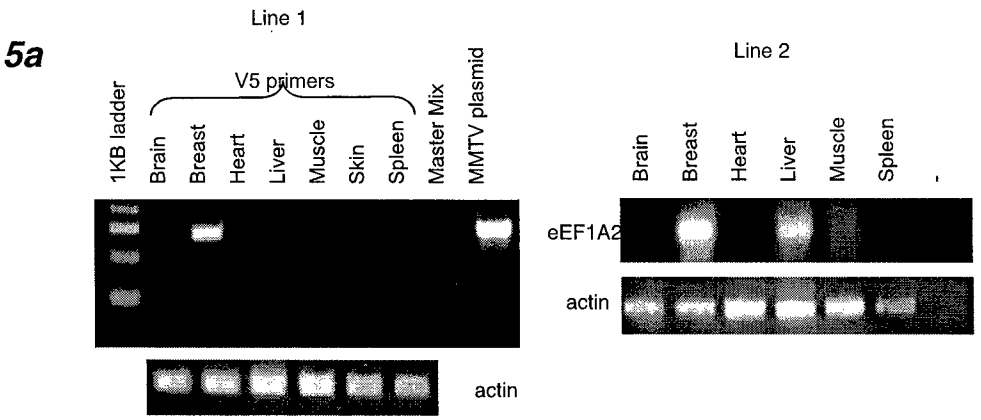


Figure 4. eEF1A2 expression protects against anoikis and leads to Akt activation. (*a*) eEF1A2 expression in BT-549 cells, BT-549-2 (▲), BT549-5 (■), BT549-8 (◆), increases their resistance to anoikis compared to parental (O) and vector alone (□) controls. Counting was performed blind and points are the mean and standard deviation of triplicate measurements. (*b*) Annexin V staining in BT 549-empty vector (control) and BT 549-8 cells grown in normal or shaking conditions from previous figure indicates greater apoptosis in the control line relative to the eEf1A2-expressing one. (*c*) Infection of BT549 cells with an eEF1A2 adenovirus increases phosphorylation of Akt as detected by Western blotting. The eEF1A2 protein is FLAG tagged for detection and the figure is a representative of three independent infections. (*d*) eEF1A2-expressing BT549 lines have higher Akt phosphorylation relative to the vector controls. The figure is representative of at least three independent experiments. Actin is a loading control.



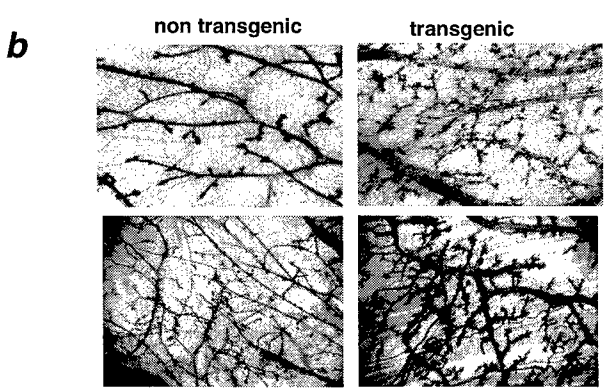


Figure 5. Breast hyperplasia in MMTV-eEF1A2 mice. (*a*) Transgene expression in two lines of eEF1A2/MMTV mice. RT-PCR was used to measure transgene expression in the indicated tissues. Actin serves as the loading control. (*b*) Transgenic mice have more nodes and increased branching relative to non-transgenic animals.

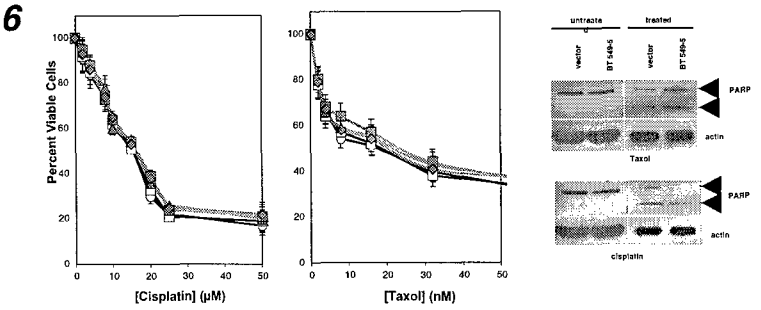


Figure 6. eEF1A2 expression has no effect on cisplatin and taxol-induced apoptosis. cEF1A2 expression in BT-549 cells, BT-549-2 (▲), BT549-5 (■), BT549-8 (◆), does not alter their resistance to death induced by cisplatin or taxol relative to parental (O) and vector alone (□) cells. The death induced by these drugs is apoptotic as shown by the induction of PARP cleavage in treated cells. The concentration of each drug was the IC_{50} of each.

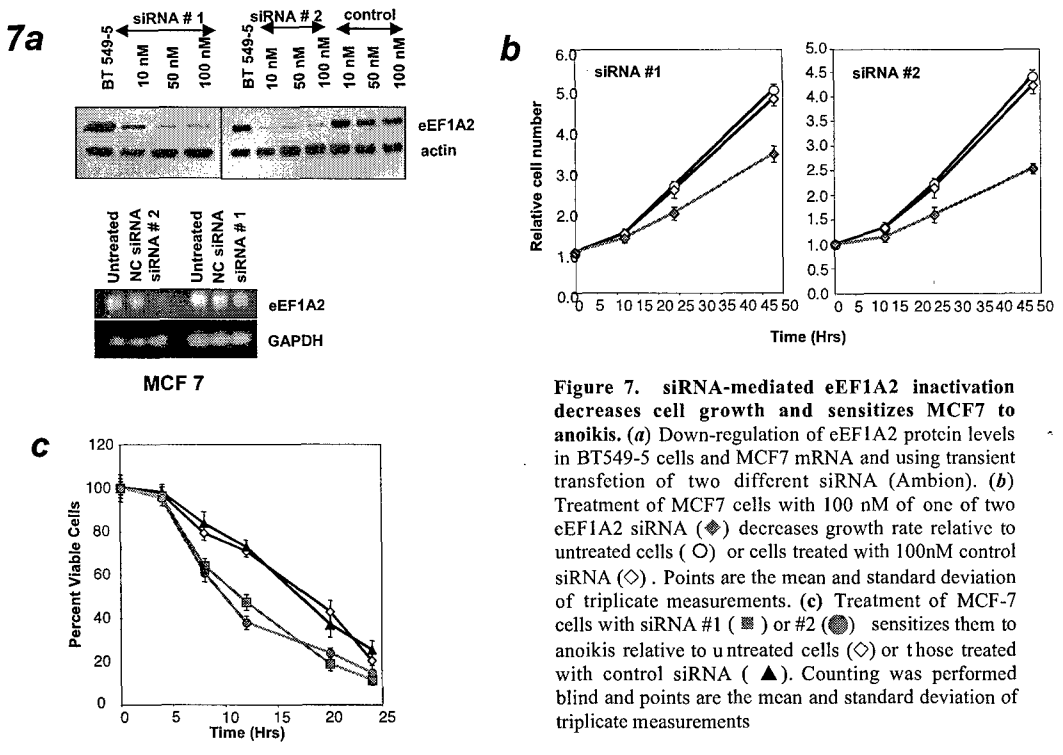
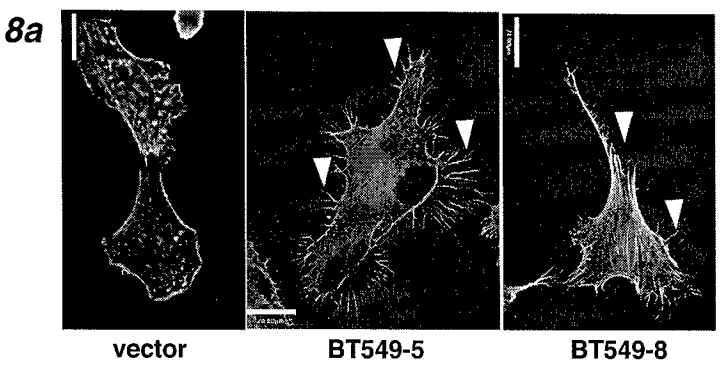
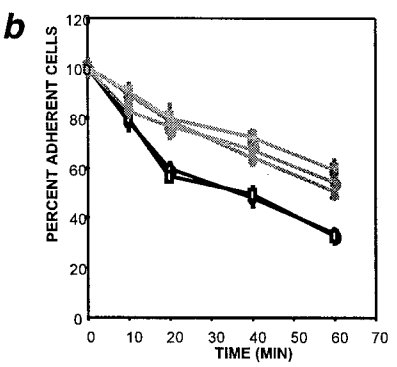


Figure 7. siRNA-mediated eEF1A2 inactivation decreases cell growth and sensitizes MCF7 to anoikis. (*a*) Down-regulation of eEF1A2 protein levels in BT549-5 cells and MCF7 mRNA and using transient transfetion of two different siRNA (Ambion). (*b*) Treatment of MCF7 cells with 100 nM of one of two eEF1A2 siRNA (◆) decreases growth rate relative to untreated cells (O) or cells treated with 100nM control siRNA (◇) . Points are the mean and standard deviation of triplicate measurements. (**c**) Treatment of MCF-7 cells with siRNA #1 (■) or #2 (●) sensitizes them to anoikis relative to untreated cells (◇) or those treated with control siRNA (▲). Counting was performed blind and points are the mean and standard deviation of triplicate measurements





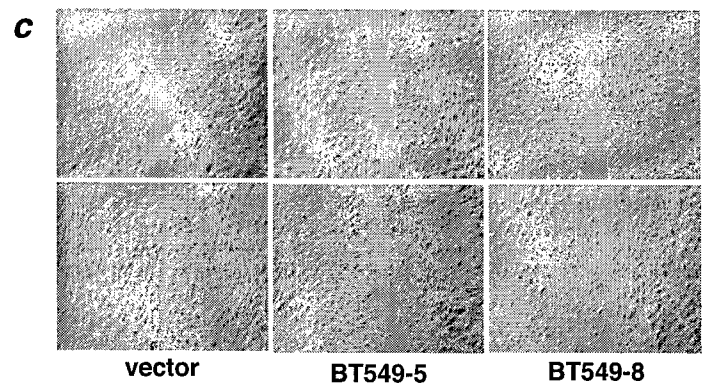


Figure 8. eEF1A2 enhances adhesion and inhibits migration. (*a*) eEF1A2 - expressing BT549 variants (BT549-5 and –8) have increased filopodia-like actin structures (yellow arrow) relative to vector only control cells. Cells were stained with phalloidin. Bar indicates 24 um. (*b*) eEF1A2-expressing BT549 cells (red lines) are more adherent to tissue culture plastic than vector and parental cells (black lines). An equal amount of all cells were plated and 2 h ours later subjected to 2100 *g* of centrifugal force. The remaining cells calculated from Hoescht 33258 fluorescence. Points are the mean and standard deviation of triplicate measurements. (*c*) eEF1A2-expressing BT549 variants (BT549-5 and –8) have reduced migration into a scratch wound than vector only control cells. Picutre is a representative oft hree independent experiments.

Appendix B. Jeganathan et al. Submitted paper.

eEF1A2 is an Akt/PKB activator that inhibits anoikis and is a marker for breast tumour recurrence*.

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Abstract

Breast cancer is the most common malignancy among North American women. The identification of factors that predict tumour recurrence risk is key to individualized disease management and to our understanding of breast oncogenesis. Here we report that high expression of eEF1A2, eukaryotic elongation factor 1 alpha 2, is a significant predictor of breast cancer relapse. eEF1A2 is highly expressed in ~30% of primary human breast tumours but not expressed in normal breast epithelium. eEF1A2 expression in breast tumours is independent of HER-2. Expression of eEF1A2 in breast cancer cells increases their growth rate and makes them resistant to anoikis (apoptotic death induced by the lack of a solid growth substrate). Inactivation of eEF1A2 by siRNA slows cell growth and sensitizes breast tumour cells to anoikis. We also find that eEF1A2 is a novel activator of the Akt/PKB kinase. Our data indicate an important role for eEF1A2 in breast oncogenesis and Akt/PKB dependent signaling.

Elongation factor eEF1A2 is one of two members of the eEF1A family of proteins (eEF1A1 and eEF1A2) that bind amino-acylated tRNA and facilitate their recruitment to the ribosome during protein translation elongation[1]. eEF1A proteins have other functions and can also induce actin[2] and tubulin[3] cytoskeleton rearrangements. Inactivation of the mouse eEF1A2 homolog, *Eef1a2*, leads to immunodeficiency and neural/muscular defects and death by 30 days of age[4,5]. We have previously reported that the eEF1A2 gene has transforming properties and is amplified and over-expressed in ~30% of ovarian tumours[6]. However, the mechanism by which eEF1A2 regulates oncogenesis is unknown. Neither is it clear whether eEF1A2 has a role in other cancers or whether its expression might have prognostic significance.

To determine whether eEF1A2 was involved in breast cancer, we analyzed eEF1A2 mRNA expression in human breast tissue derived from normal and tumour samples (n=345). We hypothesized that a role for eEF1A2 in breast oncogenesis could be inferred from high tumour-specific gene expression, an approach used to identify other candidate oncogenes[7]. eEF1A2 expression was measured from Affymetrix U133 array hybridization. eEF1A2 expression is very low in normal breast epithelium (n=22) and in fibroadenomas (n=13) (Fig 1**a**). However, approximately 30% of ductal carcinomas (73/251), lobular carcinomas (10/30) and breast tumour metastases (10/29) have eEF1A2 expression greater than 2σ above the normal tissue expression mean. This high eEF1A2 expression implicates eEF1A2 as a breast cancer oncogene.

Approximately 30% of breast tumours have high expression of the HER-2 receptor tyrosine kinase due to amplification of the HER-2 gene. Overexpression of HER-2 is a important marker in breast cancer aggressiveness[8]. We examined whether eEF1A2 expression correlated with HER-2 status. HER-2 positive (n=41) and HER-2 negative (n=210) ductal carcinomas have a similar fraction of tumours with eEF1A2 expression above normal (Fig. 1**b**) and the distribution of eEF1A2 expression between populations is not significantly different by Kolmogorov-Smirnov ($p<0.39$). This suggests that any role of eEF1A2 in breast oncogenesis is HER-2 independent.

To investigate whether eEF1A2 may have clinical relevance to breast cancer, we analyzed eEF1A2 expression in a publicly-available breast tumour expression dataset[9]. This dataset contains expression profiles from primary breast tumours annotated for tumour relapse, size, lymph node involvement, and estrogen and progesterone receptor status. We categorized tumours into two groups, eEF1A2 positive and eEF1A2 negative, based on the Affymetrix software (MAS5.0) respective call for eEF1A2 presence or absence/marginal. Mean expression in the eEF1A2 negative population is 145±86 (arbitrary units) while that of the positive population is 1609±1224 (Fig. 2**a**). All tumours in the positive population have an expression value greater than 2σ above the expression mean of the negative population; eEF1A2 expression in the two populations is different by Student's *t*-test ($p<0.0001$). Tumour size did not differ substantially between the groups (Fig. 2**b**; negative: 2.8±1.5 cm, positive: 2.9±1.6 cm). However, Kaplan Meier analysis indicates that the probability of tumour recurrence in the eEF1A2 positive population is substantially higher than in the negative cohort ($p<0.02$) (Fig 2**c**). Fifty months after diagnosis, 65% of the eEF1A2 positive group had a tumour recurrence compared to 35% in the eEF1A2 negative group. While it is likely that aggregate expression profiles of multiple genes will be the most accurate predictor of relapse[9,10], our data indicate that eEF1A2 will be useful as a single gene predictive marker for relapse.

The most important prognostic indicator for tumour relapse is axillary lymph node (LN) involvement[11]. High LN involvement (≥4 nodes) is associated with substantially increased risk of relapse relative to LN negative or LN low (1-3 nodes) cancers[12]. In our dataset, 50% of LN negative/low patients (n=55) were disease-free at 50 months, significantly more ($p<0.003$; Kaplan Meier) than the 20% disease-free in the LN high group (>3 nodes; n=10) (Fig. 2**d**). Because LN involvement is not an absolute predictor of recurrence, we determined whether eEF1A2 expression might identify LN negative/low women who were at an increased risk of relapse. In the LN negative/low group, eEF1A2 positive cancers (32/55) had an increased probability ($p<0.05$; Kaplan Meier) of recurrence relative to the eEF1A2 negative (23/55) group (Fig. 2**d**). Thus, eEF1A2 can be used to identify increased risk of relapse amongst LN negative/low patients. When tumours are segregated based on a combination of eEF1A2 negative

status and LN negative/low status, 50-month relapse in the eEF1A2-/LN low group (23/65) was ~30%, higher (p<0.007; Kaplan Meier) than the >70% relapse in the remainder (eEF1A2+ or LN $\geq$4). Thus, a combination of eEF1A2 negative and lymph node negative or low identifies a breast cancer subset with a reduced risk of relapse.

To investigate a causal role for eEF1A2 in breast oncogenesis, we investigated the effect of eEF1A2 expression or ablation in breast cancer cell lines. By northern analysis, three of five breast lines tested showed detectable eEF1A2 mRNA expression (MCF 7, BT474, and MB MDA 231) while two (BT549 and MCF10AT) showed no expression (Fig. 3**a**). This observation agrees with eEF1A2 expression previously reported from the NCI-60 cell lines[13]. We generated eEF1A2-expressing lines from two of the eEF1A2 negative lines (BT549 and MCF10AT) with eEF1A2 under the control of the cytomegalovirus (CMV) promoter. Protein expression of eEF1A2 in three independent lines of BT549 and MCF10AT cells are shown (Fig 3**b**). In both lines, eEF1A2-expressing clones showed a greater proliferation rate than the parental cells (Fig 3**c**). The BT549 and MCF10AT cell lines with the highest eEF1A2 expression (BT549-5 and MCF10AT-P5) had doubling times of 26 $\pm$ 3 hours and 17 $\pm$ 1 hours respectively, whereas the vector controls had doubling times of 47 $\pm$ 4 hours and 26 $\pm$ 2 hours. We then used two short-interfering RNAs (siRNAs) against eEF1A2 to determine what effect eEF1A2 inactivation might have on cell growth. These siRNAs decrease eEF1A2 protein in BT549 cells ectopically expressing eEF1A2 and reduce eEF1A2 mRNA in the eEF1A2-expressing MCF 7 cells (Fig. 3**d**). eEF1A2 inactivation in MCF 7 cells reduces their growth rate (Fig. 3**e**). Thus, eEF1A2 is a positive activator of breast cell proliferation.

We next investigated the role of eEF1A2 on anoikis. Anoikis, apoptotic death induced by the lack of a solid growth substrate, has an important role in controlling tumour development and metastasis[14]. We induced anoikis by shaking cultures of BT549 cells to prevent adhesion. eEF1A2-expressing BT549 cell lines consistently showed a greater fraction of viable cells than controls in adhesion-limited growth (Fig. 4**a**). After 15 hours in adhesion-limited culture, parental and vector cell survival was ~20% while

all eEF1A2-expressing cells had ~60% survival. To confirm that this death was apoptotic, we stained cells for Annexin-V. Ten hours after culture, fewer eEF1A2-expressing BT549 cells were apoptotic compared to the control (Fig. 4**b**). To confirm that this resistance to anoikis was specific for eEF1A2, we used siRNA to reduce eEF1A2 levels in BT549 cells. siRNA treatment increased the observed anoikis in the eEF1A2-expressing BT549 cells almost to wild-type cell levels (Fig. 4**c**). Moreover, both siRNAs increased cell death in MCF 7 in response to adhesion-limited culture (Fig. 4**d**), indicating that eEF1A2 inactivation increased sensitivity to anoikis. Ablation of eEF1A2 in mice leads to an increase in lymphoid apoptosis[15] and we investigated whether eEF1A2 could regulate the apoptosis induced by anticancer agents. We used cisplatin and taxol to induce apoptosis in BT549 cells. We observed that eEF1A2 expression had no effect on sensitivity to the apoptotic death induced by either drug (Fig. 4**e**). Moreover, eEF1A2 down regulation in MCF 7 cells had no measurable effect on sensitivity to cisplatin or taxol (not shown). These observations indicate that the ability of eEF1A2 to inhibit anoikis is unlikely to be due to a generalized inhibition of all forms of apoptosis.

To identify a mechanism by which eEF1A2 could enhance growth and inhibit anoikis, we investigated a role for eEF1A2 in Akt/PKB activation. Akt is a serine threonine kinase regulating multiple pathways of cell growth and apoptosis[16,17]. Infection of BT549 cells with an eEF1A2 adenovirus markedly increased phosphorylation of Akt residues serine473 (S473) and threonine308 (T308) relative to control Green Fluorescent Protein (GFP) infected cells (Fig. 4**f**). Overall protein levels of Akt did not change (Fig. 4**f**). Phosphorylation of these residues are markers for Akt activation[18]. Increases in S473 and T308 phosphorylation were also observed in the BT549 cells stably expressing eEF1A2 (Fig. 4**g**) and no changes in overall Akt, Akt2 or PI3 Kinase protein levels were observed. These observations are consistent with the idea that eEF1A2 is a novel activator of Akt and provides an explanation for how eEF1A2 regulates cell growth and anoikis.

The high expression of eEF1A2 in breast tumours and its ability to enhance cell proliferation and inhibit anoikis suggest that eEF1A2 has a causal role in breast oncogenesis. Furthermore, we have identified eEF1A2 as a novel activator of the Akt

serine/threonine kinase. The mechanism of Akt activation by eEF1A2 will require further study. However, the association of high eEF1A2 gene expression with tumour recurrence suggests that eEF1A2 could be used to identify candidates for early aggressive therapy. The ability of eEF1A2 to identify LN negative/low patients with an increased probability of relapse may increase its utility as a prognostic marker.

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METHODS

Cell lines, transfections and infections. BT549, BT474, MB MDA 231 and MCF 7 cells were obtained from the American Tissue Culture Collection (ATCC) and grown in media as indicated. The MCF10AT cells were a gift from Dr. Janusz Rak (McMaster University) and grown in DMEM/F12 (1:1) media supplemented with 5% horse serum, 0.029 M HEPES, 10 ug/ml insulin, 20 ng/ml EGF, 0.5 ug/ml hydrocortisone, and 100ng/ml cholera toxin. All cell lines were kept in a 37 °C incubator at 5% CO_2. BT 549 and MCF 10AT cells were transfected with 4.0 ug of *EEF1A2* plasmid (V5 tagged) or empty-vector pCDNA3.1 plasmid and 10 ul of Lipofectamine 2000 (Invitrogen). For adenoviral infection, eEF1A2 was sublconed into the *EcoRV* and *XhoI* sites of pShuttle-IRES and the adenovirus manufactured by the University of Ottawa. eEF1A2 and GFP control adenovirus were used at a MOI of 200.

RNA purification and northern blotting. We obtained RNA from cell lines by lysing a 60 mm plate with 1 ml of TRIzol (Invitrogen). Total RNA (10 ug) was loaded per lane and transferred to a GeneScreen membrane. Human skeletal muscle mRNA (Stratagene) was also used. Membranes were pre-hybridized at 63°C in Church's buffer for three hours, hybridized at 63 °C overnight with an EEF1A2 probe (598 base pair *Bam*HI/*Pst*I fragment) and washed 3X at 63 °C.

Western blotting. Proteins were extracted using RIPA buffer and 20 ug of protein loaded onto polyacrylamide gels. An anti-V5-HRP antibody (Invitrogen; 1:5000 inTBST (100mM Tris-Cl, 0.9% NaCl, 0.1% Tween-20, pH 7.5)) and ECL (Amersham) were used to detect eEF1A2 expression. Antibodies were used according to the manufacturers' directions are were as follows: beta actin (Sigma), PARP antibody (USBiological), Akt, phospho-Akt, PI3K and Akt2 (Cell Signaling Technology), goat anti-mouse IgG, HRP-conjugate (Upstate Cell Signaling Solutions) and anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology).

Growth and Apoptosis assays. Cells were grown in 96-well plates. Following each time interval, cells were washed once with PBS and kept at -80°C. When all time points were completed, the plates were thawed for 20 minutes at room temperature. Following the thaw, each well was incubated with 100 ul of double distilled water for an hour. Hoescht 33258 (Sigma) staining solution (20 ug/ml; 100 ul) was added to each well, and analyzed with the FluorSTAR Galaxy fluorometric plate reader after a 10 minute incubation. For apoptosis experiments, cell lines were plated in triplicate in 96-well plates for six hours. Serial half-dilutions of the drugs were made in the appropriate media and then added to wells. Twenty-four hours post-incubation with the drugs, plates were washed with PBS and analyzed as above . For anoikis assays, cells were places in standard tissue culture dishes on an S-800 orbital shaker (VWR) in the 37°C incubator. Cell viability was analyzed every four hours by trypan blue exclusion. Cell counts were performed blind in triplicate. An Apoptosis Detection Kit (Sigma-Aldrich) was used to measure Annexin staining according to the manufacturer's instructions.

siRNA. The two *EEF1A2*-directed siRNAs were 5'GGUAUUGACAAAAGGACCATT-3' (sense strand, siRNA#1), 5'-UGGUCCUUUUGUCAAUACCTC-3' (antisense strand, siRNA#1), 5'-GGACCAUUGAGAAGUUCGATT-3' (sense strand, siRNA#2), and 5'-UCGAACUUCUCAAUGGUCCTT-3' (antisense strand, siRNA#2). siPORT Lipid transfection reagent (Ambion) was used for siRNA transfection.

Gene Expression Analysis. Gene expression data in Figure 1 was extracted from the GeneExpress database from GeneLogic (Gaithersburg, MD) for probe set 204540_at on the HG-U133A GeneChip from Affymetrix (Santa Clara, CA). Microarray data values were generated by the MAS5 signal algorithm from Affymetrix. Data were extracted for samples available as of February 2003 from various tissues under normal and disease conditions. Intraductal carcinoma samples were further classified according to their HER-2 status, using the expression of probe set 216836_s_at and a threshold of 8000, which represented a visible gap in the distribution of expression values. eEF1A2 expression data from Fig. 2 were obtained from Huang et al.[9] for tumors with relapse or >45 month follow-up using hybridization to the U95Av2 Affymetrix GeneChip array and the 35174_i_at probeset. Of the 65 tumours analsyzed, 57 are infiltrating ductal carcinomas, one each are Papillary, Apocrine and Cribriform carcinomas, and the remaining 5 are classified as Other.

Figure Legends

Figure 1. eEF1A2 expression increased in breast tumours. (*a*) eEF1A2 gene expression in normal breast tissue, tumours and metastasis. The horizontal line indicates the 2σ value above the mean of the distribution of expression in normal tissue(*b*) eEF1A2 expression in HER-2 positive and negative intraductal carcinomas.

Figure 2. eEF1A2 is a marker for breast tumour recurrence. (*a*) Value of eEF1A2 expression in eEF1A2 positive (●) and negative (●) breast tumours. The horizontal line indicates the 2σ value above the mean of the distribution of eEF1A2 expression value in the eEF1A2 negative group. (*b*) Tumour size in the eEF1A2 positive (●) and negative (●) groups. (*c*) Percent of disease-free patients vs time. Green line: eEF1A2 negative; red line: eEF1A2 positive. (*d*) Percent of disease-free patients vs time. Green line: low LN; red line: high LN. (*e*) Percent of disease-free (low LN) patients vs time. Green line: eEF1A2 negative; red line: eEF1A2 positive. (*f*) Percent of disease-free patients. Green line: LN low and eEF1A2 negative; red line: other.

Figure 3. eEF1A2 enhances cell proliferation. (*a*) eEF1A2 mRNA expression, measured by northern blot in breast cancer cell lines. RNA staining of the membrane served as a loading control (*b*) Protein expression of eEF1A2 in independent clones of BT549 and MCF10AT cells, measured by Western blotting. Actin expression serves as a loading control (*c*) Ectopic expression of eEF1A2 leads to increased growth rates in BT549 and MCF 10AT cell lines. eEF1A2-expressing clones are shown in red symbols, BT549-2 (▲), BT549-5 (■), BT549-8 (◆), and controls in open symbols, parental (○) and vector alone (□). Points are the mean and standard deviation of triplicate measurements (*d*) Down-regulation of eEF1A2 protein levels in BT549-5 cells and MCF7 mRNA and using two different siRNAs. (*e*) Treatment of MCF 7 cells with 100 nM of either of the eEF1A2 siRNAs (◆) decreases growth rate relative to untreated cells (○) or cells treated with 100nM control siRNA (◇) . Mean and standard deviation of triplicate measurements are indicated.

Figure 4. eEF1A2 expression protects against anoikis and leads to Akt activation. (*a*) eEF1A2 expression in BT549 cells, BT549-2 (▲), BT549-5 (■), BT549-8 (◆), increases their resistance to anoikis compared to parental (O) and vector alone (□) controls. Counting was performed blind and points are the mean and standard deviation of triplicate measurements. (*b*) Annexin V staining in BT549-empty vector (control) and BT 549-8 cells grown in normal or shaking conditions for 10 hours from previous figure. (*c*) Treatment of eEF1A2-expressing BT549-8 cells with siRNAs targeted to eEF1A2 (●) increase cell sensitivity to anoikis relative to untreated BT549-8 cells (■) or BT549-8 cells treated with control siRNA (▲). Anoikis levels are reduced to levels of vector only BT549 cells treated with no siRNA (□), eEF1A2 siRNA (●) , or control siRNA (O) . (*d*) Treatment of MCF 7 cells with siRNA #1 (■) or #2 (●) sensitizes them to anoikis relative to untreated cells (◇) or those treated with control siRNA (▲). (*e*) eEF1A2 expression in BT-549 cells, BT-549-2 (▲), BT549-5 (■), BT549-8 (◆), does not alter their resistance to death induced by cisplatin or taxol relative to parental (O) and vector alone (□) cells. The death induced by these drugs is apoptotic as shown by the induction of PARP cleavage in treated cells. The concentration of each drug was the IC_{50} of each. (*f*) Infection of BT549 cells with an eEF1A2 adenovirus increases phosphorylation of Akt as detected by western blotting. The eEF1A2 protein is FLAG tagged for detection and the figure is a representative of three independent infections. (*g*) eEF1A2-expressing BT549 lines have higher Akt phosphorylation relative to the vector controls. The figure is representative of at least three independent experiments. Actin is a loading control.

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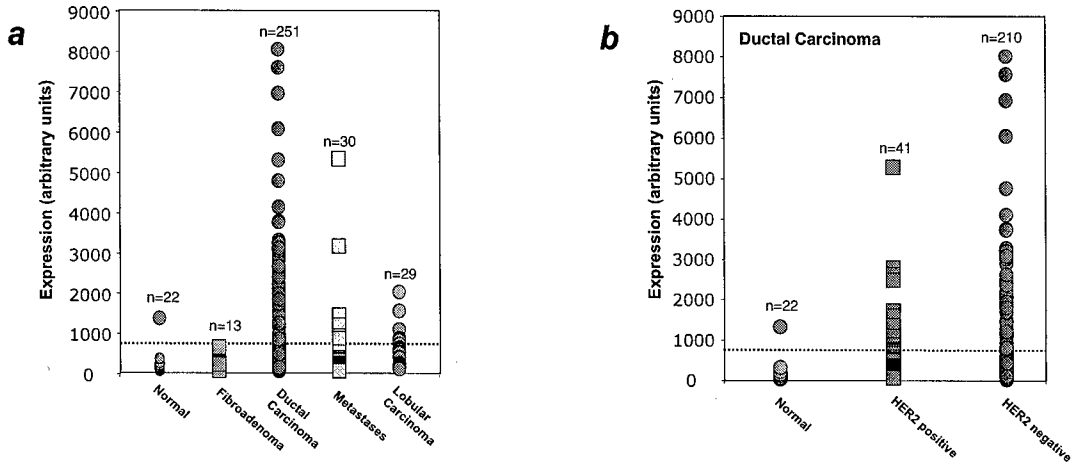
Figure 1



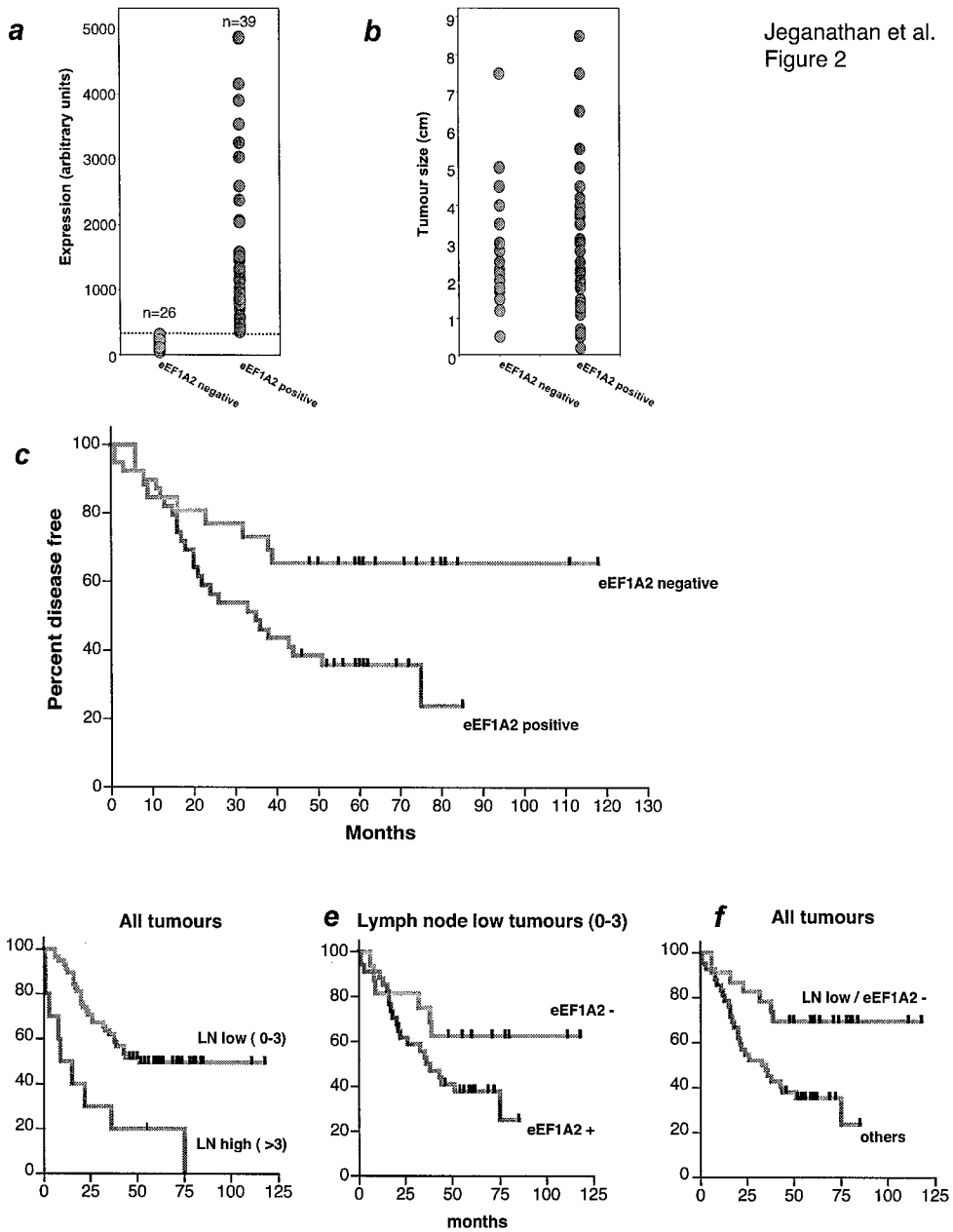


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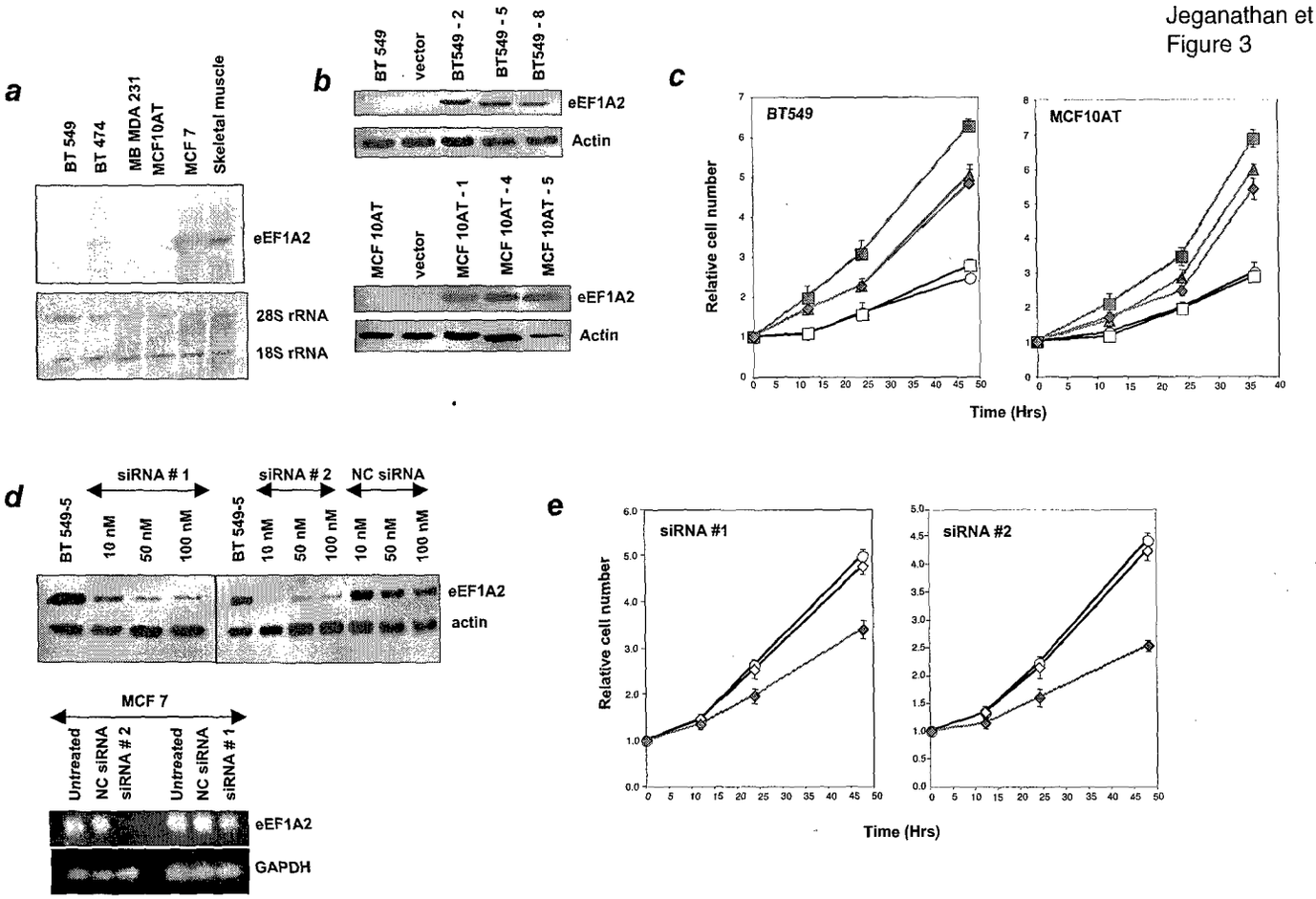
Figure 2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Skeletal muscle
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18S rRNA
b
BT 549
vector
BT549 - 2
BT549 - 5
BT549 - 8
eEF1A2
Actin
MCF 10AT
vector
MCF 10AT - 1
MCF 10AT - 4
MCF 10AT - 5
eEF1A2
Actin
c
BT549
MCF10AT
Relative cell number
Time (Hrs)
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siRNA # 1
siRNA # 2
NC siRNA
BT 549-5
10 nM
50 nM
100 nM
eEF1A2
actin
MCF 7
Untreated
NC siRNA
siRNA # 2
siRNA # 1
eEF1A2
GAPDH
e
siRNA #1
siRNA #2
Relative cell number
Time (Hrs)

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Figure 4